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A method for production of N^{α}-benzyloxycarbonyl-aminoadipate-δ-semialdehyde with amine oxidase from *Aspergillus niger*

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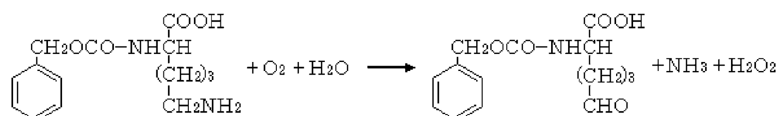
Abstract

N^{α}-Benzyloxycarbonyl-L-lysine (N^{α}-Z-L-lysine) and N^{α}-Z-D-lysine were oxidized with amine oxidase from *Aspergillus niger* according to the following scheme. The apparent K_m values for N^{α}-Z-L-lysine and N^{α}-Z-D-lysine were 50 and 70 mM, respectively. The optimum pH and temperature for N^{α}-Z-L-lysine oxidation were pH 7.5 and 45 °C, respectively. N^{α}-Z-L-Aminoadipate-δ-semialdehyde (N^{α}-Z-L-AASA) and N^{α}-Z-D-aminoadipate-δ-semialdehyde (N^{α}-Z-D-AASA) were efficiently produced from N^{α}-Z-L-lysine and N^{α}-Z-D-lysine, respectively, in the presence of catalase. Thus, amine oxidase from *A. niger* was useful for the production of N^{α}-Z-L-AASA and N^{α}-Z-D-AASA.



N^{α}-Z-L-Lysine or N^{α}-Z-D-Lysine N^{α}-Z-L-AASA or N^{α}-Z-D-AASA

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Keywords: Amine oxidase; *Aspergillus niger*; N^{α}-Z-L-Lysine; N^{α}-Z-D-Lysine; N^{α}-Z-L-Aminoadipate-δ-semialdehyde; N^{α}-Z-D-Aminoadipate-δ-semialdehyde

1. Introduction

L-α-Aminoadipic acid (L-α-AAA) and related compounds are a component of natural β-lactam antibiotics and widely acknowledged to be a raw material for the chemical synthesis of new antibiotics and functional peptides [1–3]. Recently, a microbial method for L-α-AAA production has been developed using recombinant cells containing L-lysine 6-aminotransferase (EC 2.6.1.36) [4] and Δ-1-piperideine-6-carboxylate dehydrogenase [5]. In this method, L-lysine was first converted into L-α-aminoadipate-δ-semialdehyde (L-α-AASA) by L-lysine 6-aminotransferase, and then the resulting product was converted into L-α-AAA by Δ-1-piperideine-6-carboxylate dehydrogenase. Since L-α-AASA is useful for the production of L-α-AAA and L-pipecolic acid, an enzymatic method for L-α-AASA production has also been developed with L-lysine 6-dehydrogenase (EC 1.4.1.–) [6]. However, this enzymatic method has some drawbacks such as the requirement of a cofactor regeneration system. N^{α}-Z-L-AASA is also useful as a precursor for

Abbreviations: MBTH, 3-methyl-2-benzothiazolinone hydrazone; TOOS, *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline, sodium salt, dihydrate; N^{α}-Z-L-Lysine, N^{α}-benzyloxycarbonyl-L-lysine

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production of N^{α}-Z-L-aminoadipic acid (N^{α}-Z-L-AAA). However, chemical synthesis of N^{α}-acyl-L-AASA was very complicated and its recovery was very low [7]. Recently, we revealed that N^{α}-Z-L-lysine was converted into N^{α}-Z-L-aminoadipic acid via N^{α}-Z-L-AASA by the reaction with mycelia from *Aspergillus niger* AKU 3302, and that conversion of N^{α}-Z-L-lysine into N^{α}-Z-L-AASA was catalyzed by an amine oxidase (EC 1.4.3.6) [8]. These results indicate that this amine oxidase might be useful for production of N^{α}-Z-L-AASA. As an oxidase reaction has the advantage of not requiring a cofactor regeneration system, in the present study we investigated a new method for the production of N^{α}-Z-L-AASA and N^{α}-Z-D-AASA with the amine oxidase from *A. niger*.

2. Materials and methods

2.1. Chemicals

L-Lysine, D-lysine and L-α-AAA were purchased from Wako Pure Chemicals (Osaka, Japan). N^{α}-Z-L-Lysine and N^{α}-Z-D-lysine were obtained from Calbiochem–Novabiochem (Läufelfingen, Switzerland) and Fluka Chemie (Buchs, Switzerland), respectively. N^{α}-Acetyl-L-lysine, N^{α}-butoxycarbonyl-L-lysine (N^{α}-Boc-L-lysine), N^{α}-Z-L-asparagine and N^{α}-Z-L-glutamine were purchased from Sigma–Aldrich Japan (Tokyo, Japan). N^{α}-Z-L-Arginine and N^{α}-Z-L-AAA were obtained from Sanyo Fine (Osaka, Japan). Crystalline amine oxidase from *A. niger* [9] was kindly provided by Professor O. Adachi of Yamaguchi University (Yamaguchi, Japan). All other chemicals used were of analytical grade and commercially available.

2.2. Assay of enzyme activity

Enzyme activity was spectrophotometrically assayed at 30 °C by measuring the formation rate of hydrogen peroxide. The reaction mixture (0.95 ml) was composed of 5 or 50 mM substrate and a color reagent consisting of 0.122 mg of 4-aminoantipyrine, 0.643 mg of TOOS, and 6.7 units of peroxidase per ml of 0.1 M potassium phosphate, pH 7.0. The reaction was started by the addition of 50 μl of enzyme solution, and the formation of hydrogen peroxide was followed at 30 °C for 5 min by measuring the absorbance change at 555 nm. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of one micromole of hydrogen peroxide per min by the substrate oxidation.

2.3. Standard reaction for N^{α}-Z-L-AASA production

The reaction mixture (1.0 ml) containing 50 mM N^{α}-Z-L-lysine and 12 units of amine oxidase in 0.2 M potassium phosphate, pH 7.5, was incubated at 25 °C for 4 days with shaking (120 strokes per min). The supernatant obtained by centrifugation at 20,000 × *g* for 5 min was used for analysis of the reaction products.

2.4. Analysis of reaction products

The reaction products from N^{α}-Z-L-lysine were separated by TLC with a Silica gel 60 plate (Merck, Darmstadt, Germany) or HPLC with a TSK-Gel DEAE-5PW column (Tosoh, Tokyo, Japan) under the same conditions as in our previous report [8]. The aldehyde group of the reaction product was analyzed with 3-methyl-2-benzothiazolinone hydrazone (MBTH) [8]. The concentrations of hydrogen peroxide and ammonia were assayed with the same color reagent as the enzyme assay and an Ammonia Test Wako kit (Wako Pure Chemicals, Osaka, Japan), respectively.

2.5. Determination of N^{α}-Z-L-AASA and N^{α}-Z-D-AASA

N^{α}-Z-L-Lysine, N^{α}-Z-D-lysine, and the reaction products from N^{α}-Z-L-lysine and N^{α}-Z-D-lysine were analyzed by HPLC with a TSK-Gel DEAE-5PW column at a flow rate of 0.8 ml/min at 40 °C. These compounds were eluted with water for 5 min, followed by increasing the NaCl concentration to 0.3 M with a linear gradient for 10 min, and then by 0.3 M NaCl for 10 min. The elution peaks of these compounds were detected at 210 nm, and their concentrations were calculated by the peak area.

3. Results

3.1. Substrate specificity

Using *n*-butylamine, L-lysine, D-lysine, L-glutamine, L-asparagine, L-arginine, three kinds of N^{α}-acyl-L-lysines, N^{α}-Z-L-arginine, N^{α}-Z-L-asparagine, N^{α}-Z-L-glutamine, and N^{α}-Z-D-lysine, substrate specificity of amine oxidase was assayed under standard assay conditions for enzyme activity. Among these compounds, *n*-butylamine was the optimum substrate, and N^{α}-acyl-L-lysines and N^{α}-Z-D-lysine were also oxidized, whereas other compounds were not (Table 1). Of the N^{α}-acyl-L-lysines, oxidation of N^{α}-Z-L-lysine was faster than that of N^{α}-Boc-L-lysine and N^{α}-acetyl-L-lysine (Table 1). The apparent K_m values for N^{α}-Z-L-lysine and N^{α}-Z-D-lysine were estimated to be 50 and 70 mM, respectively.

3.2. Identification of N^{α}-Z-L-lysine or N^{α}-Z-D-lysine reaction with amine oxidase

Since N^{α}-Z-D-lysine was oxidized with amine oxidase, 20 mM N^{α}-Z-D-lysine was incubated with 14 units of amine oxidase at 30 °C for 2 days, and the reaction product was analyzed by HPLC with a TSK-Gel DEAE-5PW column. The peak of N^{α}-Z-D-lysine eluting at 3.4 min decreased, and one peak was newly detected at 24.1 min (data not

Table 1
Substrate specificity of amine oxidase from *A. niger* AKU 3302

Substrate	Relative activity (%)
n-Butylamine	100
N^{α}-*Z*-L-Lysine	4.9
N^{α}-*Z*-D-Lysine	3.1
N^{α}-Boc-L-Lysine	1.4
N^{α}-Acetyl-L-lysine	0.5
N^{α}-*Z*-L-Glutamine	0
N^{α}-*Z*-L-Asparagine	0
N^{α}-*Z*-L-Arginine	0
L-Lysine	0
D-Lysine	0
L-Glutamine	0
L-Asparagine	0
L-Arginine	0

Oxidase activity was assayed under standard assay conditions with 50 mM substrates and 0.033 units of amine oxidase.

shown). Since this elution time was the same as that of N^{α}-*Z*-L-AASA, the aldehyde group of this eluate was analyzed with MBTH. Derivative 2 with MBTH of this eluate showed two adsorption maxima at 620 and 660 nm (adsorption spectra were the same as those of N^{α}-*Z*-L-AASA in [8]), indicating that the product eluted at 24.1 min contained an aldehyde group. Thus, N^{α}-*Z*-D-lysine was also oxidized into N^{α}-*Z*-D-AASA with amine oxidase and the resulting product, N^{α}-*Z*-D-AASA, was eluted at the same time as N^{α}-*Z*-L-AASA by HPLC with a TSK-Gel DEAE-5PW column. Since it was revealed that N^{α}-*Z*-L-lysine and N^{α}-*Z*-D-lysine were oxidized into N^{α}-*Z*-L-AASA and N^{α}-*Z*-D-AASA, respectively, with amine oxidase, other reaction products were assayed. The formation of hydrogen peroxide and ammonia was also confirmed in the reaction mixture of both N^{α}-*Z*-L-lysine and N^{α}-*Z*-D-lysine, and their concentrations increased in parallel with the substrate consumption (data not shown). These results indicate that amine oxidase from *A. niger* oxidized the ε-amino group of N^{α}-*Z*-L-lysine and N^{α}-*Z*-D-lysine, and N^{α}-*Z*-L-AASA and N^{α}-*Z*-D-AASA were formed from N^{α}-*Z*-L-lysine and N^{α}-*Z*-D-lysine, respectively, according to Scheme 1.

3.3. Effects of pH and temperature on N^{α}-Z-L-lysine oxidation

The effect of pH on N^{α}-*Z*-L-lysine oxidation was investigated under standard assay conditions for enzyme activity, except that the reaction pH varied between pH 6.0 and 8.5. The maximum oxidation rate was obtained at pH 7.5 (Fig. 1). When N^{α}-*Z*-L-lysine was incubated between 20 and 50 °C for 5 min, the maximum reaction rate occurred around 40–45 °C at pH 7.0 (Fig. 2).

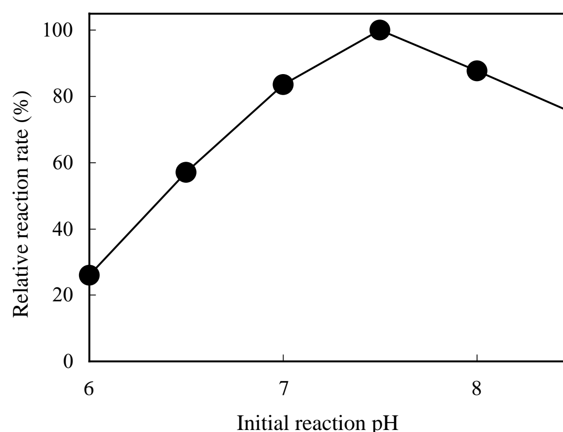


Fig. 1. Effect of pH on N^{α}-*Z*-L-lysine oxidation. The reaction rate of N^{α}-*Z*-L-lysine oxidation was assayed under standard assay conditions, except that reaction pH varied between pH 5.0 and 8.5 with 0.2 M potassium phosphate buffer.

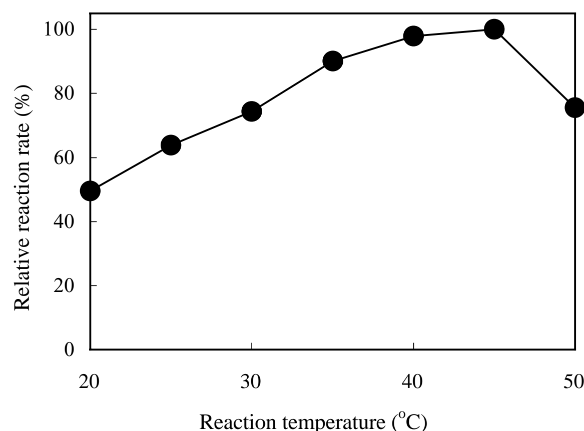
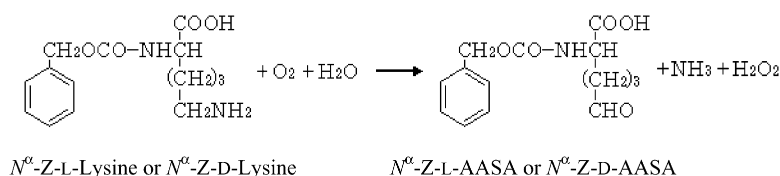


Fig. 2. Effect of temperature on N^{α}-*Z*-L-lysine oxidation. The reaction rate of N^{α}-*Z*-L-lysine oxidation was assayed under standard assay conditions, except that reaction temperature varied between 20 and 50 °C.

3.4. Optimum conditions for N^{α}-Z-L-AASA production

On the basis of the results of the initial reaction rate for N^{α}-*Z*-L-lysine oxidation, the optimum conditions for N^{α}-*Z*-L-AASA production were investigated. When N^{α}-*Z*-L-lysine was incubated between 20 and 40 °C for 4 days at pH 7.5, N^{α}-*Z*-L-AASA production was highest at



Scheme 1. Oxidation of N^{α}-*Z*-L-Lysine and N^{α}-*Z*-D-Lysine with amine oxidase from *A. niger* AKU 3302.

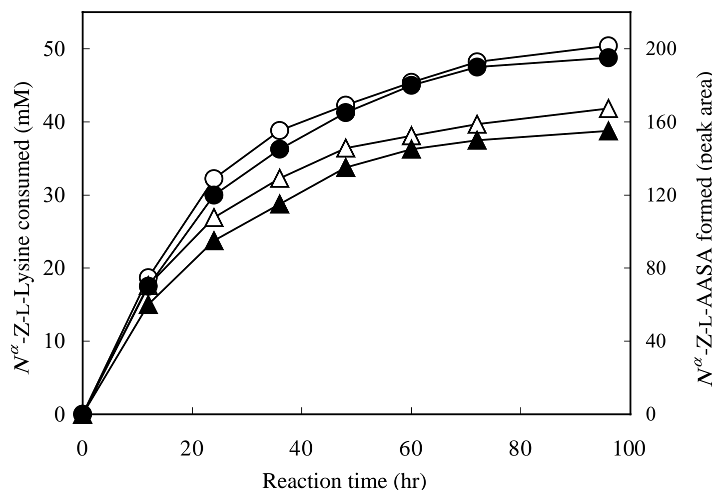


Fig. 3. Effect of catalase on N^{α}-Z-L-AASA production. N^{α}-Z-L-Lysine (50 mM) was incubated with 12 units of amine oxidase and 600 units of catalase in 0.1 M potassium phosphate buffer, pH 7.5, at 25 °C for 4 days. Open circles, N^{α}-Z-L-lysine consumed in the presence of catalase; closed circles, N^{α}-Z-L-AASA formed in the presence of catalase; open triangles, N^{α}-Z-L-lysine consumed without catalase; closed triangles, N^{α}-Z-L-AASA formed without catalase.

25–30 °C (data not shown), which was much lower than the optimum temperature in the initial reaction rate. When N^{α}-Z-L-lysine was incubated between pH 6.0 and 8.5 for 4 days at 25 °C, N^{α}-Z-L-AASA production was highest at pH 7.5, which was the same as the optimum pH in the initial reaction rate. These results indicate that N^{α}-Z-L-AASA production was affected by the heat stability of amine oxidase. On the basis of these results, 50 mM N^{α}-Z-L-lysine was incubated with 12 units of amine oxidase at 25 °C for 4 days at pH 7.5. Approximately 80% of N^{α}-Z-L-lysine was consumed, and the formation of N^{α}-Z-L-AASA increased in parallel to the consumption of N^{α}-Z-L-lysine (Fig. 3). Since it has been reported that alcohol oxidase was inactivated by hydrogen peroxide produced by the oxidation of substrate [10], the effect of catalase on N^{α}-Z-L-AASA formation was investigated by adding 600 units of catalase into the reaction mixture containing 50 mM N^{α}-Z-L-lysine and 12 units of amine oxidase. The reaction rates of both N^{α}-Z-L-lysine consumption and N^{α}-Z-L-AASA formation exceeded that of the reaction without catalase. In addition, product amounts of N^{α}-Z-L-AASA increased approximately 1.3 times by the addition of catalase (Fig. 3).

3.5. Production of N^{α}-Z-D-AASA

As N^{α}-Z-D-lysine was also oxidized by amine oxidase, 50 mM N^{α}-Z-D-lysine was incubated with 14 units of amine oxidase and 600 units of catalase at 25 °C for 4 days at pH 7.5. N^{α}-Z-D-Lysine was almost completely consumed, and the formation of N^{α}-Z-D-AASA and ammonia increased in parallel to the consumption of N^{α}-Z-D-lysine (Fig. 4). Thus, N^{α}-Z-D-AASA was also efficiently produced from

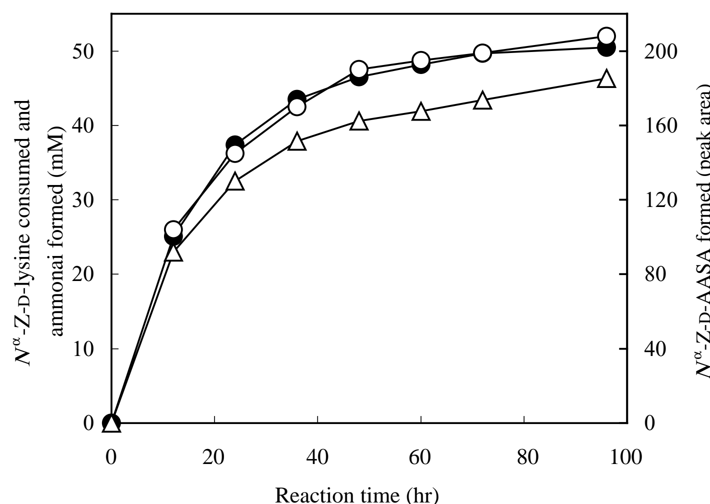


Fig. 4. Production of N^{α}-Z-D-AASA. N^{α}-Z-D-Lysine (50 mM) was incubated with 14 units of amine oxidase and 600 units of catalase in 0.1 M potassium phosphate buffer, pH 7.5, at 25 °C for 4 days. Open circles, N^{α}-Z-D-lysine consumed; closed circles, N^{α}-Z-D-AASA formed; open triangles, ammonia formed.

N^{α}-*Z*-D-lysine by amine oxidase under the same conditions as N^{α}-*Z*-L-AASA production.

4. Discussion

L-α-AAA and its related compounds have become increasingly important as raw materials for chemical synthesis of new antibiotics and physiological peptides. Chemical methods for the synthesis of L-α-AAA, N^{α}-acetyl-DL-α-AAA, L-α-AASA, and N^{α}-acyl-L-AASA have been reported, but until now they have been very complicated and their recoveries were very low [7,11,12]. Since L-α-AASA is useful for the production of L-α-AAA and L-pipecolic acid, an enzymatic method for the production of L-α-AASA has also been reported using L-lysine and L-lysine ε-dehydrogenase [6]. However, this method has some drawbacks, such as requirement of a cofactor regeneration system. Recently, we found that N^{α}-*Z*-L-lysine was converted into N^{α}-*Z*-L-AAA via N^{α}-*Z*-L-AASA by reaction with mycelia of *A. niger*, and that conversion of N^{α}-*Z*-L-lysine into N^{α}-*Z*-L-AASA was catalyzed by amine oxidase [8]. Therefore, we investigated in detail the reaction of amine oxidase from *A. niger* with N^{α}-*Z*-L-lysine or its related compounds. This amine oxidase oxidized N^{α}-*Z*-L-lysine, N^{α}-Boc-L-lysine and N^{α}-acetyl-L-lysine, but did not oxidize L-lysine, N^{α}-*Z*-L-glutamine, N^{α}-*Z*-L-asparagine or N^{α}-*Z*-L-arginine. N^{α}-*Z*-D-lysine was also oxidized, whereas D-lysine was not. Thus, amine oxidase from *A. niger* catalyzed the oxidative deamination of ε-amino group of N^{α}-acyl-L-lysine and N^{α}-acyl-D-lysine, and N^{α}-acyl-L-AASA and N^{α}-acyl-D-AASA were produced from N^{α}-acyl-L-lysine and N^{α}-acyl-D-lysine, respectively. Since the chiral N^{α}-acyl-AASA can be produced by amine oxidase without the need for a cofactor regeneration system, the optimum conditions for N^{α}-*Z*-L-AASA production were investigated using N^{α}-*Z*-L-lysine as a substrate. N^{α}-*Z*-L-AASA was efficiently produced at pH 7.5 and 25 °C in the presence of catalase. Under these optimum conditions, more than 95% of N^{α}-*Z*-L-lysine was oxidized, and a corresponding amount of N^{α}-*Z*-L-AASA was produced. N^{α}-*Z*-D-AASA was also efficiently produced under the same conditions. Thus, the present enzymatic method with amine oxidase was simple and superior to the previous chemical methods for production of N^{α}-acyl-AASA.

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